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Progress Report

RESISTANCE OF PINES TO BARK BEETLES

Studies on Toxicity of Resins, Forced Attacks, and Analysis of Ponderosa Pine Terpenes, 1962

By Richard H. Smith/ Entomologist

NOT FOR PUBLICATION

U. S. DEPARTMENT OF AGRICULTURE, FOREST SERVICE
PACIFIC SOUTHWEST FOREST AND RANGE EXPERIMENT STATION

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Progress Report

RESISTANCE OF PINES TO BARK BEETLES

Studies on Toxicity of Resins, Forced Attacks, and Analysis of Ponderosa Pine Terpenes, 1962

By

Richard H. Smith, Entomologist

SUMMARY

Research on the resistance of pines to bark beetle attack was continued in 1963. The field work was carried out at the Institute of Forest Genetics at Placerville where several improvements were made in facilities. Air conditioning was installed in two rooms, an air cooler was added to the insectary, a single-pan microanalytical balance and a gas chromatograph were purchased for the laboratory.

Procedures developed in previous work were used to recheck the vapor toxicity of pine resin vapors to Dendroctonus brevicomis and D. monticolae. Both beetles were reared from naturally infested material. There was a general agreement with previous results with two exceptions. Lodgepole pine resin vapor caused an increase in the mortality rate of D. brevicomis but it did not quite reach a significant level; in previous work a significant increase was just barely reached. Sugar pine resin vapor caused a significant increase in the mortality rate of D. monticolae; in previous work this resin had never caused an increase, let alone a significant one. No reason has been found for this striking change.

Differences were found in the vapor toxicity of individual terpenes to both D. brevicomis and D. monticolae. The vapor toxicity of the terpenes, nearly pure and all constituents of ponderosa pine resin, can be ranked in decreasing order for both beetles as follows: limonene > Δ_3-carene > β-pinene > α-pinene.

The pure monoterpene distillate of the resin of different ponderosa pines was used in equal volumetric dosages in vapor toxicity tests with D. brevicomis. In general, vapors with the higher concentration of limonene plus Δ_3-carene caused a greater increase in mortality than vapors containing a lower concentration of these two terpenes.

Differences again were found between D. monticolae and D. jeffreyi in their mortality rate in the presence of certain pine resin vapors.

Considerable effort, largely unsuccessful, was devoted to the development of a procedure which would enable regulated forced attack by D. brevicomis to be made on ponderosa pine.

A gas chromatographic study was made on ponderosa pine resin terpenes. Very little, if any, difference in the results of analysis could be attributed to:

1. Type of sample preparation.
2. Length of storage time of sample.
3. Type of column used in the analysis.
4. Time of resin flow from a wound.
5. Place (height and circumference) of obtaining resin from a tree.
6. Time of season for obtaining resin.
7. Dormant or active season of growth.
8. Year of obtaining resin.

Appreciable differences were found for average values of the terpene composition of plots located in the central Sierra Nevada. Large differences were found among trees within plots; the average values followed by the minimum and maximum, in parentheses, of 65 trees which were analyzed are as follows: Δ_3-carene 36.2 percent (trace to 82.5 percent), β-pinene 26.4 percent (trace to 57.5 percent), limonene 14.5 percent (trace to 30.7 percent), myrcene 13.3 percent (4.6 to 27.5 percent), α pinene 6.3 percent (1.5 to 13.3 percent), β-phellandrene 1.8 percent (0.3 to 3.7 percent), unknown 1.5 percent (0.0 to 3.1 percent), and a trace of camphene and heptane.

An inverse relationship seems to exist between the amounts of Δ_3-carene and β-pinene in a tree.

INTRODUCTION

Research on the resistance of pines to bark beetles took a somewhat different course in 1963 than in previous seasons. Though vapor toxicity studies were continued and expanded to include variations between ponderosa pines, two new major studies were started: (1) forced attack experiments and (2) gas chromatographic analysis of pine terpenes.

Several improvements were made in the facilities at the Institute of Forest Genetics and in instrumentation. Air conditioning was installed in the basement laboratory so that a constant temperature could be maintained during vapor toxicity tests. Air conditioning was also installed in the general biology laboratory which greatly improved the operation of the gas chromatograph. An air cooler was

installed in the insectary which enabled a 10° to 15° F. depression of the midday temperatures. This cooler also permitted a partial regulation in the rate of adult emergence.

Two additional instruments were acquired: an Ainsworth single-pan analytical balance and a gas chromatograph. The balance greatly increased the number of samples which could be handled and decreased their processing time. The gas chromatograph is an Aerograph A-90-P using a Brown "Electronik" recorder with a disc integrator. With appropriate conditions the time for analysis of the terpenes in a 4 µl. sample can be made in 30 minutes; conventional methods require at least 1 quart and 30 days.

The field work, including a portion of the gas chromatographic analysis, was done at the Institute. Additional chromatographic analyses and the statistical analyses were made in Berkeley. Gordon Frankie, an entomology major at the University of California in Berkeley, assisted in the field work.

The report will be given in two parts. The first will be devoted to vapor toxicity and forced attack experiments, the second to gas chromatographic studies of ponderosa pine resin.

VAPOR TOXICITY STUDIES

Previous research (Smith 1961a) reported on the development of a technique for determining the toxicity of resin vapors to adult Dendroctonus. Using this technique it was found (Smith 1961b) that, in general, beetles can tolerate saturated resin vapors of host resin but not of nonhost resin. There were suggestions in this work that different broods or sources of D. brevicomis differ in their reaction to the same resin vapors and that the same brood might react differently to the resin vapors of the same tree extracted at different times of the year. Differences in the effect of resin vapor on adult beetles were found between different trees of the same species, though the differences were not significant. These results suggest checking prior work and expanding the vapor toxicity studies to include tests of the resin vapor of different trees, particularly of trees having quite different terpene composition.

Experiments were, therefore, run throughout the course of the season to (a) recheck the results of previous season's test with larger replication, (b) determine the toxicity of some of the individual terpene components of ponderosa pine, and (c) test the effect of the vapors of the low temperature distillate of resin from ponderosa pines with large variation in their terpene makeup. Dendroctonus brevicomis Lec. was used in all three types of experiments while D. monticolae Hopk. was used in only (a) and (b); D. jeffreyi Hopk. was compared with D. monticolae in a few tests.

PROCEDURES

Brood material was collected and handled in much the same way as in 1961. About two cords of D. monticolae brood logs, 18-inch length, were obtained from a ponderosa pine stand at Crystal Bay, Nevada, in mid-June. Brood development at that time ranged from fully grown larvae to callow adults. All material was put directly into the insectary; about one cord of D. jeffreyi brood material was cut from Jeffrey pine near Fallen Leaf Lake in mid-June and put directly into the insectary. D. brevicomis brood material was obtained throughout the summer by stripping bark from infested ponderosa pines near Placerville. Bark was removed when the brood had reached the late larval or pupal stage. Part of the bark was put in the insectary while the rest was put in the coldroom and moved to the insectary as needed. The air cooler in the insectary depressed midday temperatures by as much as 10° to 15° F. at highs of 95° to 85° F.

Beetles were collected twice daily at about 8 a.m. and 8 p.m. in individual #000 gelatin capsules and were held at 35° F., usually for 24 to 48 hours, until used in tests. Therefore, the average beetle had been emerged for 6 hours and held for 1 to 2 days at 35° F. prior to use. Beetles were equitably allocated to all replicates in a test, according to size by ocular estimate. Replicates were randomly assigned to treatments. The row-block order established by this assignment was maintained for the course of the test and the subsequent statistical analysis.

The procedures used to obtain and handle resin and other test materials and the trees which were the sources of these materials were the same as those used in 1961. Ponderosa resin distillates were obtained by processing fresh resin in a Hickman still at 40° C. for 20 to 24 hours.

Fresh resin was collected with a microtapping device, and for test purposes the samples of the resin were apportioned with a 10 cc. pipette into 3 cc. vials. One sample, usually weighing 200 to 300 mg., was placed in each fumigation chamber. The latter consisted of an airtight 150 cc. screwcap jar in which beetles in fumigation cells were also placed. Samples of each test material were serially weighed to obtain the weight of the vapor produced in the chamber during the treatment period. The sample weights were considered representative of the material in the test. After the test was completed, the resin samples were subjected to additional processing to obtain data on percent volatility at various conditions of time and temperature in an open atmosphere. Resin distillates and individual terpenes were apportioned with a 10 µl. microsyringe and were injected directly into the 150 cc. chamber to eliminate any handling. These two types of materials volatilize so rapidly that any handling, such as via the standard 3 cc. resin vial, could result in appreciable loss of material. A few samples

were carefully and quickly processed to determine a factor to convert volume to weight.

Vapor toxicity was determined as in previous studies by confining the beetles to individual cells within the fumigation chamber (150 cc. jar) with substance to be tested. Each test was replicated. A replicate of D. brevicomis consisted of 12 beetles in one jar (unless otherwise stated); a replicate of D. monticolae or D. jeffreyi consisted of 10 beetles in two jars. Beetles were kept in the chamber for 4 to 7 days. At the end of the treatment period the jars were uncapped and a count made of the living and dead beetles in each replicate. The criterion for a dead beetle was the lack of movement when agitated. Beetles were held in a nonresinous atmosphere for observation on subsequent mortality counts after the treatment period.

Results of the vapor toxicity experiments were analyzed with electronic computer by making an F-test of the mortality data for each replicate. Duncan's multiple-range testing was used whenever an F-value was obtained which was greater than "F" at 95 percent. The multiple-range testing was used to discriminate treatments which differed at the 95 percent level.

DENDROCTONUS BREVICOMIS

Pine Species and Hybrids

The recheck experiment was composed of 11 replicates of beetles for each of the species and hybrids used in previous work. A 5-day treatment period was used with the standard conditions. The results (table 1) substantiate previous findings except those for lodgepole pine. The latter, a nonhost for D. brevicomis, failed to cause an increase in mortality at the 95 percent confidence level, though it did so at the 90 percent level. This result is not too surprising since in the 1961 work a 95 percent level of significance was achieved in one test but not in another. Nevertheless, in all tests with lodgepole, mortality was greater than with the untreated check and other host resins.

Host pine resin vapors--Coulter and ponderosa--caused no significant increase in mortality. Nonhosts and hybrids containing one nonhost--knobcone, Monterey, knobcone X Monterey, Jeffrey X ponderosa, and Jeffrey X Coulter--caused a significant increase in mortality. The resin vapor of sugar pine, a nonhost, caused no significant increase, as in the case of previous work.

Table 1.--Mortality of D. brevicomis in vapor toxicity tests using saturated vapors of indicated resins for 5 days at 74° F.

Resin	Vapor saturation	Mortality
	mg./150 ml.	Percent
Coulter	2.2	10.6
Sugar	2.3	12.9
Control	--	14.4
Ponderosa	3.3	16.7
Lodgepole	2.5	1/ 22.0
Knobcone X Monterey	4.7	1/ 25.8
Knobcone	5.0	1/ 31.8
Monterey	5.6	1/ 34.8
Jeffrey X Ponderosa	8.8	1/ 41.7
Jeffrey X Coulter	10.2	1/ 52.3

1/ Significantly greater than control at 95 percent confidence level.

Individual Terpenes

Tests with individual terpenes included four of those found in ponderosa pine--α-pinene, β-pinene, Δ_3-carene, and limonene--and heptane, a nonterpene and the major component of Jeffrey pine resin vapor. These five compounds were reasonably pure, running somewhere near 95 percent; impurities were usually the other terpenes. Their complete volatility enabled volumetric dosages to be used to obtain the desired weight to volume concentration in the 150 cc. chamber. Volumetric dosages of 4 µl. and 8 µl. were used for all terpenes and 5 µl. and 10 µl. for n-heptane. These amounts are roughly equivalent to 3.2 mg. and 6.4 mg. per 150 cc. respectively. The lower concentration is a bit higher than what can be expected from whole fresh resin of ponderosa pine; the latter is much higher. Six replicates were used with a 4-day treatment period at 74° C.

The results (table 2) show striking differences between the terpenes. α-pinene was nontoxic with either dosage. β-pinene, Δ_3-carene, and limonene were increasingly toxic in that order. Heptane was nontoxic at the low dosage but was toxic at the high one. This suggests that the resin vapor of Jeffrey pine, which is nearly pure n-heptane, is toxic because it can reach a vapor saturation greater than 10 µl. Previous work with fresh resin of Jeffrey pine produced vapor saturation in excess of 25 µl.

Table 2.--Mortality of D. brevicomis in vapor toxicity tests using given dosages of individual terpenes for 4 days at 74° F.

Material	Dosage per 150 ml.[1]	
	4 µl.	8 µl.
	Percent	
Control	4.2	4.2
α-pinene	8.3	9.7
n-heptane	6.9	[2] 16.7
β-pinene	[2] 18.1	[2] 56.9
Δ3-carene	[3] 36.1	[2] 62.5
Limonene	[3] 40.3	[3] 70.8

[1] 5 µl. and 10 µl. for n-heptane.

[2] Significantly greater than control at 95 percent confidence level.

[3] Significantly greater than control and β-pinene at 95 percent confidence level.

These results also help to explain the vapor toxicity findings with sugar pine and Monterey pine resins. Each of these resins is chiefly composed of α-pinene and β-pinene (table 3), yet sugar pine resin vapor is nontoxic while Monterey is toxic. However, the fact that sugar pine resin vapor saturates at about 3 µl. while Monterey resin vapor saturates at about 8 µl. can account for the difference. Why the vapor of these two should saturate at such different values, even though they contain nearly the same volatile components, presents an important question. One rather nebulous explanation is the existence of a physical relationship between the solid and liquid fractions of resin which in some way controls the rate of volatility and possibly the vapor saturation equilibrium. One additional possibility is that the percent volatility (Smith 1963) of Monterey pine resin is about 25 percent at 75° F. while sugar pine is only about 2 percent. However, this is a complex problem which is beyond the scope of the current work.

Table 3.--Approximate percent α-pinene and β-pinene in the terpene fraction of sugar pine and Monterey pine resin as recorded by different workers

Source of data	Monterey		Sugar	
	α	β	α	β
	Percent			
Mirov (1961)	30	70	70	5
	70	30	--	--
Bannister (1959)	25	75	--	--
Smith	50	50	66	25

Natural Terpene Mixtures

Results of vapor toxicity tests with the individual terpenes, plus the knowledge (reported in the section on resin studies) that there is considerable intraspecific variation between ponderosa pines in their terpene composition, suggested a test using the pure monoterpene extract of ponderosa pines differing widely in this respect. The use of the monoterpene fraction alleviated the problems associated with the handling of fresh resin and also enabled the use of equal dosages.

Four ponderosa pines were selected (table 4) which varied in their terpene composition and whose monoterpene fraction had been obtained from fresh resin by the Hickman molecular still at 40° C. for 24 hours. Volumetric dosages of 2 µl., 3 µl., and 4 µl. per 150 cc. were applied, giving respectively approximately 1.6, 2.4, and 3.2 mg. These are dosages which cover the range which might be obtained with fresh resin. The middle dosage is near average while the other two represent quite low and quite high dosages for ponderosa pine. In setting up these tests the dosages were applied directly to the chambers; the small resin vials were not used. Five replicates of 10 beetles each were used with a 4-day treatment at 74° F.

Table 4.--Monoterpene composition of four ponderosa pines[1/] used in vapor toxicity tests

Tree designation	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown
	Percent						
1-IFG	7.8	35.3	34.7	9.1	9.5	1.1	1.9
14-Joseph Creek	1.5	.1	82.5	11.1	1.5	1.4	1.9
1-Crystal Bay	4.4	12.8	43.6	12.5	22.6	2.8	1.3
2-IFG	5.8	14.9	50.8	11.0	12.7	2.0	2.8

[1/] No. 1-IFG (Institute of Forest Genetics) is the tree used in practically all previous vapor toxicity studies; No. 2-IFG was used for some of the early exploratory studies; No. 14-Joseph Creek and No. 1-Crystal Bay are phenotypically resistant to D. monticolae attack.

The results (table 5) show that the low dosage was nontoxic. At the middle dosage the three monoterpene extracts having the larger proportion of Δ_3-carene and limonene were significantly more toxic than either the untreated or the monoterpene extract with the smaller proportion of these two terpenes. This suggests that, other factors being equal or noncritical, the beetle population would have a much more difficult time in overcoming trees with a high proportion of Δ_3-carene and limonene. However, one additional observation is that the resin with the highest Δ_3-carene-limonene composition (No. 14-Joseph Creek) did not cause the greatest mortality. This might be due to the low limonene content or the action of such phenomena as synergism, potentiation, or masking.

All monoterpene extracts were toxic at the high dosage, and here the expected one--No. 14 Joseph Creek--caused the highest mortality. The results with the high dosage may explain, in part, the infrequent occurrence in previous work of significant toxicity by whole resin of No. 1-IFG, which was the basic tree in all previous vapor toxicity tests; i.e., vapor toxicity will result if the vapor saturation can be increased.

Table 5.--Mortality of D. brevicomis in vapor toxicity tests using given dosages of monoterpene extract of the indicated pines for 4 days at 74° F.

Tree designation	Dosage		
	2 µl.	3 µl.	4 µl.
	Percent		
Control	26	18	1/26
1-IFG	30	1/20	2/50
14-J. Creek	34	1/36	1/74
1-C. Bay	34	1/40	1/56
2-IFG	38	1/42	2/70

1/ Significantly greater than control at 95 percent level of confidence.

2/ Significantly greater than control and 1-IFG at 95 percent level of confidence.

DENDROCTONUS MONTICOLAE AND D. JEFFREYI

Pine Species and Hybrids

The work with D. monticolae paralleled, in general, that with D. brevicomis except that the toxicity of the monoterpene extract of different ponderosa pines was not determined.

The recheck experiment with D. monticolae used the same resins, a 7-day treatment at 74° F., and six replicates of beetles. The results (table 6) agree quite well with previous work except those for sugar pine. In all previous work the resin vapor of sugar pine, a host of the beetle, had never caused a significant increase in mortality and usually caused no increase at all. There is no clear reason for the results obtained this season. The tree was the same as before; the tests were conducted under as nearly identical conditions as possible; the beetles were handled in the same manner; and they were obtained from the same forest area at Crystal Bay.

The other three host resin vapors (ponderosa, Coulter, and lodgepole pine) caused no significant increase in mortality, while the two nonhost resin vapors and their hybrid (knobcone, Monterey, and knobcone X Monterey pine) did cause a significant increase. The resin vapor of one nonhost X host hybrid, Jeffrey X ponderosa pine, just made the significant level while that of another one, Jeffrey X Coulter pine, failed to cause a significant increase. In previous work the latter also failed to cause a significant increase while the former gave mixed results, sometimes significant toxicity and sometimes not.

Table 6.--Mortality of D. monticolae in vapor toxicity tests using saturated vapors of indicated resins for 7 days at 74° F.

Pine resin	Vapor saturation	Mortality
	mg./150 ml.	Percent
Control	0.0	36.7
Jeffrey X Coulter	11.8	38.7
Ponderosa	2.5	45.0
Lodgepole	2.1	46.7
Coulter	2.1	46.7
Jeffrey X ponderosa	9.9	55.0 1/
Knobcone	5.2	61.7 1/
Monterey	5.4	71.7 1/
Sugar	2.0	71.7 1/
Knobcone X Monterey	5.2	75.0 1/

1/ Significantly greater than control at 95 percent level of confidence.

Individual Terpenes

A rather heavily replicated test was made to compare the effects of the vapors of host, nonhost, and hybrid resin and nearly pure β-pinene on both D. monticolae and D. jeffreyi. Sufficient fresh resin was used to obtain saturation; 10 µl. of β-pinene were used to obtain approximately 8 mg. of vapor.

The results (table 7) show that D. monticolae was unaffected by the resin vapor of either ponderosa pine or the Jeffrey X ponderosa pine hybrid. The vapors of Jeffrey pine resin were extremely toxic to the beetle. The 8 mg. dosage of β-pinene was likewise quite toxic.

Table 7.--Mortality of D. monticolae and D. jeffreyi in vapor toxicity tests with resins and a resin derivative for 4 days at 74° F.

Material	Vapor concentration	D. monticolae	D. jeffreyi
	mg./150 ml.	Percent	
Control	0.0	14	24
Ponderosa	2.4	18	1/ 45
Jeffrey X ponderosa	8.0	21	25
Jeffrey	28.6	1/ 100	1/ 45
β-pinene	8.0	1/ 52	1/ 71

1/ Significantly greater than control at the 95 percent level of confidence.

Only the vapors of the Jeffrey X ponderosa hybrid were nontoxic to D. jeffreyi; the three other materials, ponderosa pine and Jeffrey pine resins and β-pinene, were toxic. Jeffrey pine resin vapor had not caused significant toxicity to this beetle before. However, in the previous tests the resin vapor saturated at nearly 20 mg. while in this test it was almost 30 mg., thus as with D. brevicomis it appears that in high enough concentrations host resins may be toxic.

It should be noted that by comparison D. monticolae was much more adversely affected by Jeffrey pine resin vapor than was D. jeffreyi, and that D. monticolae was unaffected by ponderosa pine resin vapor while D. jeffreyi was. Thus the general notions about these two bark beetles and resins are still reasonably sound.

A second test was made with D. monticolae and D. jeffreyi to compare their reaction to 7 mg. of β-pinene and 7 mg. n-heptane; this dosage was obtained with 8.5 µl. and 10 µl. respectively. The standard procedure was used with six replicates of beetles for 4 days at 74° F. As in other tests with resin derivative, the materials were applied directly to the 150 cc. chamber and the resin vial was not used.

The results (table 8) were a bit surprising since both beetles reacted similarly to each treatment. However, as in previous work, the results do point out the importance of concentration in vapor toxicity studies.

Table 8.--Mortality to D. monticolae and D. jeffreyi in vapor toxicity tests using 7 mg. per 150 ml. of the indicated materials for 4 days at 74° F.

Treatment	D. monticolae	D. jeffreyi
	Percent	
Control	12	17
n-heptane	18	17
β-pinene	1/ 38	1/ 36

1/ Significantly greater than control at the 95 percent level of confidence.

A final test with D. monticolae used the same dosages and individual terpenes as were used with D. brevicomis. Unfortunately, the replication, four replicates of 10 beetles each, was not as large as desired. The test ran for 4 days at 74° F. The results (table 9) again show the importance of concentration since n-heptane was not nearly as toxic as the terpenes. But probably of most interest is limonene which was significantly more toxic than either β-pinene and α-pinene. Δ_3-carene was not significantly more toxic than β-pinene. It will be recalled that it was more toxic than β-pinene to D. brevicomis. It is also interesting to note that the ranking of the materials is the same for both beetles.

Table 9.--Mortality of D. monticolae in vapor toxicity tests using indicated dosage of the materials for 4 days at 74° F.

Material	Dosage 1/	
	4 µl.	8 µl.
	Percent	
Control	12.5	12.5
n-heptane	12.5	2/ 12.5
α-pinene	22.5	2/ 25.0
β-pinene	2/ 32.5	2/ 55.0
Δ_3-carene	2/ 35.0	3/ 57.5
Limonene	3/ 55.0	3/ 85.0

1/ n-heptane was at 5 µl. and 10 µl.

2/ Significantly greater than control at 95 percent level of confidence.

3/ Significantly greater than control and β-pinene at 95 percent level of confidence.

The obvious test of D. monticolae with the four monoterpene extracts, which were used with D. brevicomis, was not made because of the lack of beetles late in the season when these tests were made.

FORCED ATTACK STUDIES

Vapor toxicity results which have been obtained to date have been based on laboratory tests and, therefore, could be affected by the artificial conditions of testing, which may be quite different than the interactions of the natural factors of the environment. The existence of such possibilities injects a doubtful tone into any conclusions, and the need for the development of field testing procedure to substantiate the laboratory results is quite apparent.

In the past there have been two approaches to field studies: (1) detailed observation and measurement of existing infestations, and (2) forced-attack studies. The latter procedure was selected for the season's work because it offers some opportunity of regulating the intensity of beetle attack. Previous forced-attack studies relied on the caging of a large number of beetles, or a large amount of brood material from which beetles would soon emerge, about the basal 6 to 12 feet of a pine. In general, the value of results of these older studies was questionable because they did not produce uniformly successful results. The lack of control of beetle activity and the lack of adequate quantitative criteria for evaluation prevented reliable interpretation.

The procedure devised for the season's work attempted to overcome these shortcomings by (1) regulating the intensity of attack and by (2) seeking objective criteria for measurements of results.

DEVELOPMENT OF TECHNIQUE

Regulation of attack intensity was attempted by starting individual beetles in a geometric attack pattern on an area of bark on the trunk of the test tree. The bark area to be used was first made quite smooth with a draw knife. A template of the particular attack design was used to locate each attack spot. Four procedures were tried before one was found suitable for establishing individual beetle attacks. These are briefly discussed as follows:

1. A 1 mm.-diameter hole was drilled straight into the bark for 1 to 2 millimeters. One-half of a 000 gelatin capsule containing a recently collected beetle was placed over the hole and held in place with a straight pin. Two trees were selected, and densities of 9 and 25 attack attempts per square foot for 1 square foot were used. The flow of resin from the attack spot was used as the criterion of an active attack. At 24 hours there were 2 and 7 active attacks for the 9 and 25 attack attempts respectively. There were no additional active attacks

after the first 24-hour period. At 2 weeks the attacks were examined and none were active or were found to be successful, based on the presence of eggs. In fact, in most cases the beetle barely reached the cambium. No gallery was more than ½-inch long, and the beetle was usually encrusted within the crystallized resin. A necrotic area developed around the attack spot on the outer face of the phloem but rarely penetrated to the cambium. The maximum size of the necrotic area on the outer face of the phloem was 1 inch by 2 inches with the greater dimension parallel with the grain. This technique was considered unsuitable.

2. The second method of establishing an attack was to drill a 5/32-inch hole 1 to 2 millimeters into the bark at a slight downward slant. A freshly collected beetle was placed in the hole which was then plugged with a small piece of lumite screening. Again densities of 9 and 25 attack attempts per square foot for 1 square foot were used; at 24 hours there were 2 and 13 active attacks respectively for the two densities. The 2-week examination revealed essentially the same results as those for method 1. In addition it was noted that some beetles bored outward through the screening rather than into the bark. This method was also considered unsuitable.

of course!

3. The third method employed a 23/64-inch diameter hole about 3 millimeters deep into the bark at a slight downward slant. The open end of a gelatin capsule half just fitted into this size hole. A freshly collected beetle was placed in the gelatin capsule half. Attack attempts of 9 and 25 per square foot for 1 square foot were used on each of two trees. The capsule was presumably held in place with a small tack. However, during the first 24 hours many of the capsules became dislodged and the test was considered a failure.

4. The three previous methods showed the need for reliable means of attaching the capsule to the bark and for a greater incidence of active attacks. Attachment was greatly improved by the use of two small strips of rubberized friction tape, each of which was fastened to the capsule and the bark in the form of an L. Also the open end of the capsule was given a slanting angle so that it could be placed against the bark with the upper surface of the capsule at an angle of about 60° rather than 90° from the bark as in previous methods. This slant of the capsule kept the beetle from sliding away from the bark.

A change was also made in determining the sex of the beetle. In the previous tests the larger beetles were used with the assumption that most would be females; it was felt that any of the standard ways of sexing would entail too much handling. To obtain beetles of known sex 6-inch-long bolts were cut from fresh ponderosa pine 6 to 8 inches in diameter. Pole-size trees or tops from freshly logged trees

! is that how you get them?!

AN ILLUSTRATION NEEDED

served as a source of these bolts. At one end of a bolt, a 1-inch ring of the circumference was made smooth by removing almost all the bark. Small v-shaped notches were made through the phloem at about 3/4-inch intervals. The opening of the notch was on the cross sectional surface, and the point was one-eighth to one-fourth inch in from the end. A band--either thin acetate or heavy kraft paper--was tightly fastened around the smooth bark so that at least one-half inch was over the smoothed ring of bark and at least one-half inch projected beyond the end of the bolt. The bolt was placed upright on the unimproved end, and freshly collected beetles were placed on the cross section surrounded by the band. The number of beetles used was $2\frac{1}{2}$ times the number of notches. A petri dish was placed over the ring to hold the beetles on the cross section. The prepared bolts were placed in a darkroom. (If left in a light room, the beetles gathered at the light side and interfered with each other's activities.

GOD WHAT A COMPLICATED WAY OF GETTING BEETLES OF KNOWN(?) SEX.

Under the conditions just described a female readily bored into the phloem via a notch; and was usually, but not always, joined by a male. A period of 2 to 4 days was sufficient for most females to construct galleries. The galleries were typical of D. brevicomis, and eggs were laid within 1 day after the start of the gallery. The female was at the front end of the gallery; the male some distance behind her. They were recovered by carefully stripping away the rest of the bark. Paired and unpaired females were held in individual gelatin capsules until placed on a tree.

are these "normal" beetles?

Using this method for obtaining beetles of known sex, a test was conducted much like the previous three tests, but with slanted capsules attached by friction tape. The test consisted of 9 and 25 attack attempts per square foot for 1 square foot at a height of both 3 and 16 feet on the tree. The latter was about one-third of the ~~way~~ up ~~from~~ the base of the crown. At 24 hours there were 6 and 20 active attacks respectively at the 3-foot height but only 1 and 11 respectively at the 16-foot height. Poor fitting of the capsules at this latter height was responsible for some loss of beetles.

TESTS WITH REGULATED FORCED ATTACKS

The four tests just described concluded that part of the forced attack study devoted to the development of a method for establishing an attack. The procedure described last ("conditioned" beetles in slanted capsules attached with rubberized friction tape) was selected as the most suitable, though it had not been adequately proven.

Good reporting of failures.

Single Set of Attacks

Both height of attack and condition of beetle were tested on one tree. Six heights, from base of crown to within 10 feet of the tip, were selected. At each height, a 1-square-foot area was prepared and divided laterally. Sixteen newly emerged beetles were placed in one-half of the area, and 16 "conditioned" beetles were placed in the other half. The 24-hour count of active attacks was as follows:

Number of active attacks out of 16 attempts at 24 hours

Height (Feet)	Newly emerged (Number)	"Conditioned" (Number)
22	2	10
25	6	4
28	2	4
31	5	5
35	2	5
39	0	3
	17	31

really

The 31 active attacks by "conditioned" beetles were much greater than the 17 by newly emerged beetles. Therefore, "conditioned" or "sexed" beetles were used in subsequent tests. The test results do not permit a sound conclusion on the effect of height; therefore, a 3- to 6-foot height was selected for the later tests largely because of convenience.

A second test, using a single set of attacks, was made to compare the east and west sides of trees. Three trees were selected, and 25 attack attempts per square foot for 1 square foot were started on both the east and west sides. No detailed data were taken, and only the general observation made that there was little difference between the east and west sides so far as success of attack was concerned, but there appeared to be considerable difference between trees. The lack of what might be called successful attacks suggested additional changes in the procedure.

Repeated Sets of Attacks

In previous tests all attempts to start attacks on a given tree were made within a period of 24 hours. Since these efforts were unsuccessful, tests were conducted using repeated sets of attack attempts within the same general bark area of the tree over a period of several days or weeks.

(a) Six trees were selected for the first test using 25 attack attempts per square foot for 1 square foot on each tree at a

Free choice by beetle + intensity is the secret.

one bee sting won't kill most people either

height of 4 feet. The first set of beetles was put on the tree on July 20 without measurement of results. However, it was found that the gelatin capsules had to be swung back away from the attack hole as soon as resin started to flow; otherwise the resin would collect in the capsule to prevent normal action of the beetle. The second set was established on July 31 by placing each attack approximately 1 inch above the first set. Again only general observations were made. The third set was installed on August 31 using the square foot immediately below that used for the first two sets. Observations of active attacks, based on the flow of resin from the entrance hole, showed considerable differences between the trees. The number of active attacks for the six trees were 6, 7, 9, 12, 16, 17. However, 4 weeks later no successful attacks could be found. All attacks were similar to those described earlier.

(b) This test used 10 trees, a density of 30 attack attempts per square foot for one-third of a square foot (the one-third foot was approximately 12 inches by 4 inches), and 4 sets of attempts each occupying the one-third square foot immediately below the previous one. The first set of beetles was started at about a $4\frac{1}{2}$-foot height. The dates on which each set was installed were July 31, August 16, August 31, and September 3. The data on the 24-hour active attacks for the third and fourth sets of beetles are as follows:

Number of active attacks out of 10 attempts

Tree number	Third set	Fourth set
1	8	7
2	6	7
3	4	1
4	5	4
5	1	6
6	9	6
7	5	8
8	7	6
9	1	4
10	10	6

The detailed examination 4 weeks after the last set showed no successful attacks. Attacks which had been considered active at 24 hours were much like those described for earlier tests.

(c) In this experiment the factor of height was tested by starting the beetles at both 3 and 12 feet above ground. A density of 36 attempts per square foot for one-fourth of a square foot on three trees was used. (The one-fourth square foot was 6 inches square.) Different sets of beetles were put on as follows:

August 1: One-fourth square foot at both heights.
August 16: One-fourth square foot at 12 feet using the same spots as used on August 1.
August 31: Both heights, using the previous spots at 12 feet and both the previous spots and also the one-fourth square foot immediately below the previous ones at 3 feet.

The number of active attacks 24 hours after the last date was as follows:

Tree number	Height (Feet)	Number of active attacks out of 9 attempts
1	12	1
2	12	4
3	12	0
1	3 (upper set)	4
2	3 (upper set)	2
3	3 (upper set)	2
1	3 (lower set)	3
2	3 (lower set)	3
3	3 (lower set)	4

When examined, active attacks, though not successful, were similar to those reported for other tests.

Results of forced-attack studies were, generally, inconclusive and somewhat disappointing, considering the large amount of effort it took to conduct the tests. Nevertheless, much was learned which will permit better planning for future studies of this type. There were large differences between the trees used in practically all resin properties investigated; i.e., pressure, quantity of flow, quality, percent volatility, and vapor saturation. However, they mean very little since good criteria could not be found to differentiate tree effect on the beetle.

GAS CHROMATOGRAPHIC ANALYSIS OF PONDEROSA PINE RESIN TERPENES

Pine resin has long been suspected of playing an important, if not decisive, role in the resistance of pines to bark beetles.

Resin is considered a secondary plant substance and, therefore, does not reenter the basic metabolic pathways. The secondary status of resin is now being seriously questioned, however. Resin is a complex mixture of molecules and, in essence, can be considered a supersaturation of rosin in turpentine. Rosin is a mixture of various resin acids; turpentine a mixture of terpenes. Mirov (1961) summarizes the work on terpenes prior to 1959. Most of the studies of pine terpenes have been of two types: (a) bulk sample

analyses in which the resin from several trees was mixed before analysis; (b) single tree analysis. These procedures either masked individual tree variation or failed to account for it. Practically all studies were based on open-face collected resin, steam distillation, and conventional chemical methods which at best were unable to detect constituents present in small quantities. Also, these methods were slow and required as much as a quart of resin.

The use of gas chromatography for terpene analysis was mentioned briefly by Mirov (1961). Bannister et al. (1959) applied this analytical procedure to the study of hybridism in Pinus radiata and P. attenuata. Their results showed little variation in terpene composition between individual trees of these two species. Williams and Bannister (1962) analyzed the terpenes of 22 species of pines grown in New Zealand. Analyses of 21 of the species were based on a single tree sample; there was no reference to the original source of the tree.

A brief resumé has been made (Smith and Eaton, in press) of the research efforts attempting to associate resin with resistance of pines to bark beetles. Resin properties which have been investigated are quantity (Callaham 1955), pressure (Vité and Wood 1961), and quality (Smith, in press). In the latter it was shown that bark beetles were affected quite differently by resin vapors of different pine species.

In view of possible implication of terpenes in the resistance of pines to bark beetles, and the lack of adequate data on the variation in terpene composition between ponderosa pines, a study of these substances was started. Its objective was to determine variability in pine resin terpene composition which might be associated with (a) time and place of obtaining resin from the tree, (b) method of preparation and analysis of the sample, and (c) age and geographic and local differences between trees.

GENERAL PROCEDURES

Trees at eight localities in northeastern California were selected for study (fig. 4). All resins were collected with a closed-face microtap. Practically all of the microtaps were one-half inch in diameter; a few were 2 inches. Samples were prepared for analysis within 2 to 3 days after collection with few exceptions, though it was found that as much as a 1-month delay caused no change in the constituents.

Two subsamples were prepared for each collection of resin, if there was sufficient resin in the sample. Petroleum ether or alcohol was added to resin at a ratio of from one to one-third the volume of resin. The second way was to process the sample in a Hickman molecular still at 40° C. for 20 to 24 hours at atmospheric pressure, using ice to cool the condensing surface. The usual procedure

was to pour from 1 to 8 cc. of resin into the still, leaving from 0.5 to 1 cc. of the resin in the collecting vial. Ether or ethanol was added to the collecting vial and the contents transferred to a $\frac{1}{2}$-dram screwcap vial. The distillate recovered from the still was also kept in a $\frac{1}{2}$-dram screwcap vial. Samples were kept at 5° C. in a refrigerator unless being used. Only the ether extract could be made if there was less than 1 cc. of resin in the sample.

All analyses were made with the same gas chromatograph[1/] and recorder[2/] (fig. 1) under the following conditions:

(1) Mobile phase: helium with flow rates of 90 or 60 ml. per minute at the outlet port.
(2) Column: 8-feet by $\frac{1}{4}$-inch stainless steel.
    (a) Solid support: 60/80 acid-washed Chromosorb W.
    (b) Liquid support: 10 percent or 20 percent oxydipropionitrile[3/] (ODPN).
(3) Injector at 120° to 130° C.
(4) Column at 58° to 62° C.
(5) Detector at 145° to 152° C.
(6) Filament current at 200 milliamps.
(7) Volume of sample varied from 0.2 µl. to 4.0 µl.

Qualitative determinations were made in three ways:

(1) Comparison of retention times with existing data in the literature (ODPN: Klouwen and ter Heide 1962; LAC: Bernhard and Scrubis 1961; DDP: Williams and Bannister 1962).
(2) Comparison of retention times with those of known compounds using reasonably pure samples of β-pinene, α-pinene, camphene, Δ_3-carene, myrcene, and limonene. For β-phellandrene the comparison was based on the major peak of lodgepole pine.
(3) Internal standardization with mixtures of the compounds in (2).

Quantitative determination was made by internal normalization of disc integrator values. The use of known percentages of synthetic terpene mixtures proved internal normalization to be a valid procedure for monoterpenes. A typical chromatogram is given in figure 2.

1/ Aerograph: Model A-90-P with a thermal conductivity detector.

2/ Minneapolis-Honeywell: Brown "Electronik" with a disc integrator.

3/ 20 percent didecylphthlate (DDP) and 20 percent LAC-446 were also used for comparisons and for certain small parts of the work. However, oxydipropionitrile was used for all results reported except those involving column comparisons.

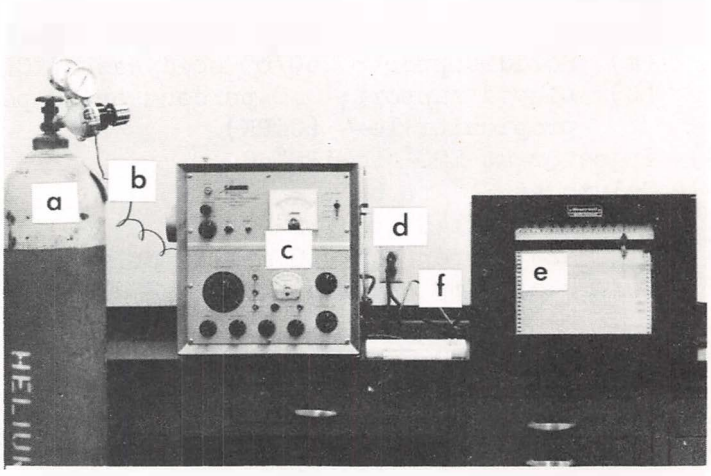

Figure 1.--Gas chromatograph with source of helium and means of recording.

a. Tank of helium.
b. Helium line to gas chromatograph.
c. Gas chromatograph.
d. Power supply.
e. Recorder.
f. Signal line from chromatograph to recorder.

BASIS FOR QUALITATIVE AND QUANTITATIVE DETERMINATIONS

Qualitative

Table 10 gives the relative retention times obtained on two different columns for the peaks of a chromatogram of the distillate of ponderosa pine resin, and of a known synthetic mixture whose constituents had been run individually beforehand. The agreement of the two columns establishes the identity of the constituents of ponderosa pine resin vapor as indicated. When the synthetic mixture was added to the liquid derivative, there was a corresponding increase in the peak area representing the given terpene without the occurrence of additional peaks or shoulders on existing peaks.

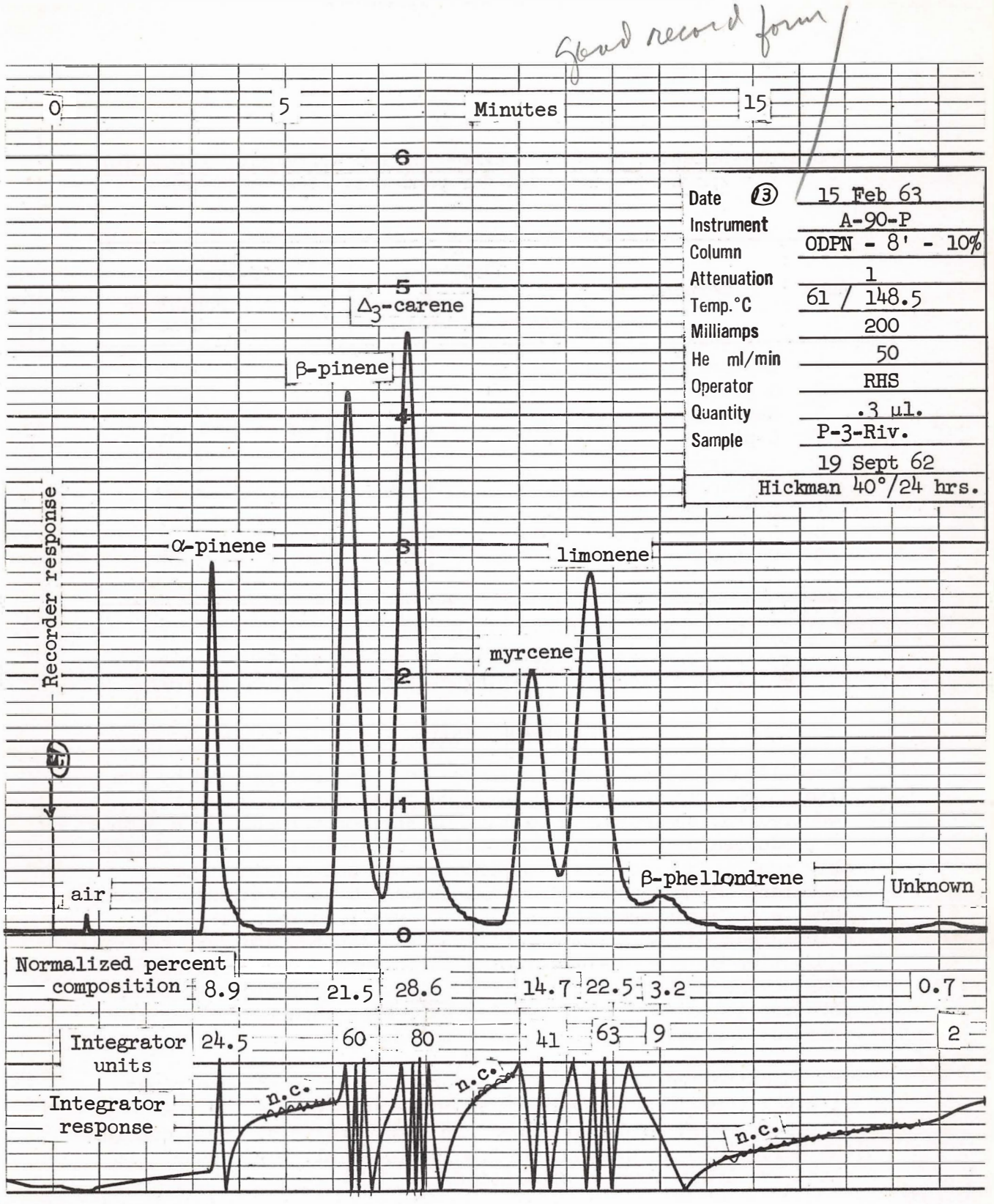

Figure 2.--Chromatogram of ponderosa pine resin showing recorder response, integrator response, integrator units for each curve area, and the internally normalized area under each curve which represents the percent composition.

Table 10.--Relative retention time[1] on two different columns for known compounds and for ponderosa pine resin vapor

Peak	Terpene	ODPN[2]		LAC-446[3]	
		Known	Ponderosa pine	Known	Ponderosa pine
1	Heptane (probably)		.29		.15
2	α-pinene	1.00	1.00	1.00	1.00
3	Camphene	1.20		1.43	
4	β-pinene	1.89	1.88	1.83	1.81
5	Δ_3-carene	2.30	2.32	2.40	2.38
6	Myrcene	3.16	3.19	2.73	2.70
7	Limonene	3.51	3.54	3.45	3.40
8	β-phellandrene	4.00	4.01	3.67	3.62
9	Unknown		5.91		5.89

[1] Based on 1.00 for α-pinene.
[2] 3.415 minutes to α-pinene at 55° C. and 60 ml./min.
[3] 10.620 minutes to α-pinene at 60° C. and 60 ml./min.

Quantitative

Three liquid supports were selected for use as suggested by the literature. The DDP column was used first, and 2½ hours were required for the complete elution of the monoterpenes in a sample of ponderosa pine resin. Qualitative determinations were reasonably good. However, clear separation was not obtained for Δ_3-carene and myrcene or for limonene and β-phellandrene. Furthermore, a column temperature of 100° C. was required. The LAC column reduced the time of elution to 45 minutes and gave better separation of the above mentioned components. The 20 percent ODPN column reduced the time to 20 minutes, permitted the temperature to be reduced to 60° C., but, most important, gave the sharpest separation of the monoterpenes.

The quantitative values obtained for different ponderosa pines with all three columns were quite comparable (table 11). A 10 percent ODPN column was used for later analysis. This enabled the helium flow to be reduced from 90 to 60 ml. per minute. It also gave a bit sharper separation.

Table 10.--Ponderosa pine resin monoterpene composition determined with different columns

Tree	Column	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown[1/]
				Percent				
10-J. Creek	LAC	6.5	31.4	46.8	8.1	7.1		-
	ODPN	5.4	25.9	51.2	7.0	6.3	2.0	2.2
1-C. Bay	LAC	5.6	13.8	43.5	14.0	23.1		-
	ODPN	4.4	12.9	43.7	13.1	22.3	3.6	T
2-IFG	LAC	7.5	13.9	49.8	12.1	14.1		2.6
	ODPN	5.0	14.6	51.8	11.2	13.1	1.9	2.3
1-IFG	LAC	8.6	37.4	34.2	10.6	9.2		
	ODPN	7.4	33.8	34.4	9.7	10.0	2.1	2.7
Ch. 5-J. Creek	DDP	1.5	3.9	72.1	9.5	12.2		
	ODPN	2.2	3.3	73.3	7.8	11.1	1.1	1.1
56-J. Creek	DDP	5.9	38.3	43.8	11.7	.4		
	ODPN	7.6	40.0	37.4	11.3	1.4	2.3	2.3
Ch. 1-J. Creek	DDP	5.4	18.2	55.4	9.5	11.4		
	ODPN	6.4	17.5	52.1	7.9	10.7	2.5	2.7

[1/] Determinations of the unknown were not made for the DDP column.

PROCEDURAL VARIATIONS

Several tests were made to determine the variation which could occur with sample collection, preparation, and analysis. These were (1) ether extract versus molecular distillate, (2) distillation time, (3) first flow of resin versus last flow from the collection wound, and (4) time of holding prepared samples.

Ether Extract Versus Molecular Distillate

The chromatographic analysis of the ether extract and the molecular distillate of samples of resin of ponderosa pines were compared. The results (table 12) show very little difference between the two types of subsample preparation. Both the extrate and the distillate should be prepared. The extract can be made very quickly and enables a large number of trees to be analyzed quite easily. However, ether and heptane are not sharply separated by the ODPN column, and a small impurity in ether comes off as a shoulder of camphene.

Table 12.--Resin terpene composition of selected ponderosa pines prepared by ether extract (Ext.) and by molecular distillation (Dis.)

Tree number 1/	Preparation	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown
		Percent						
1-Riv.	Dis.	5.9	21.0	37.6	19.6	13.2	1.2	1.4
	Ext.	5.4	19.8	38.6	17.3	14.6	1.9	2.4
3-Riv.	Dis.	9.1	22.1	28.6	15.0	21.9	2.5	.7
	Ext.	8.0	20.6	29.1	13.4	25.1	1.7	2.0
4-Riv.	Dis.	11.8	49.2	-	16.5	18.7	3.8	-
	Ext.	10.8	52.8	-	14.9	19.2	2.3	-
1-Kyz.	Dis.	6.9	28.9	19.2	16.9	25.2	1.6	1.2
	Ext.	6.3	29.5	20.1	14.4	26.2	2.2	1.2
3-Kyz.	Dis.	5.6	21.4	26.5	24.1	19.1	1.8	1.6
	Ext.	5.2	21.4	28.2	21.8	20.2	2.0	1.1
4-Kyz.	Dis.	3.8	13.8	26.5	25.5	25.5	3.0	1.9
	Ext.	4.2	14.5	27.7	23.3	27.3	1.4	1.6
1-Pyr.	Dis.	4.4	16.9	52.7	9.5	11.7	2.0	2.8
	Ext.	3.4	15.7	55.5	9.0	12.6	1.1	2.8
9-IFG	Dis.	8.0	9.0	66.5	8.2	4.5	.4	3.3
	Ext.	7.8	9.1	64.4	9.1	5.3	.7	3.7

1/ Riverton, Kyburz, Pyramid, Institute of Forest Genetics.

Distillation Time

At medium temperatures whole fresh resin may be considered azeotrophic; i.e., the constituents of a mixture of volatiles with similar boiling points vaporize in proportion to their concentration in the mixture. A test with the molecular still was made with the resin of ponderosa No. 1-IFG to check this property and to determine the effect of distillation time. Approximately 7.5 cc. of freshly collected resin were placed in the still. At 1, 2, 3, 4, 5, 9, 13, and 24 hours, the distillate was removed and analyzed, and a clean vial was placed on the collection arm of the still to receive the distillate for the next time interval. An ether extract was made of the resin also. The series of samples was reanalyzed 2 to 3 months later to see if any change had occurred in the interim. The results (table 13) show that even the first couple of drops represent the actual composition of the terpene mixture, as indicated by the values at the end of 24 hours or by the analysis of the ether extract of the resin. Thus, fresh resin of ponderosa pine is essentially azeotrophic and 20-hour molecular distillation of amounts less than 10 cc. yields a distillate which represents the true monoterpene composition. Even a 12-hour period is sufficient to extract almost all the monoterpenes.

Time of Resin Flow From Wound

The question could be raised whether the composition of the first and last flow of resin from a wound is comparable. The affirmative would be expected; thus only a small test was made by analyzing the first and second 24-hour flow of resin from standard collection wounds on five trees. The results, given in table 14, show that there is no difference between the first and second 24-hour flow of resin from ponderosa pine.

Holding Time of Prepared Samples

Analyses made at the time of preparation of the sample were compared with those made after 4 to 6 months' storage. Comparisons were made of extracts and distillates. The results (table 15) show no change during storage of either type of preparation.

WITHIN-TREE VARIATION

Tests were made to determine the possibility of time and place of within-tree variation in monoterpene composition of ponderosa pine resin.

Place on Tree

Tree No. 3 at IFG was tapped at the four cardinal points at a 3- and 60-foot height; the latter was about 10 feet from the top of the tree. The samples were analyzed on the DDP column. The results

Table 13.--Terpene composition of the molecular distillate of ponderosa pine resin at cumulative distillation time periods for freshly distilled and 3-months-old material

Hour	Cumulative amount recovered	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown
		Percent						
Fresh distillate								
1st	.2	11.0	39.5	32.3	7.9	6.2	3.1	T
2nd	.5	10.4	38.1	33.0	8.5	6.9	3.1	T
3rd	.7	9.9	38.5	33.0	8.4	7.1	3.0	.3
4th	.85	9.5	38.0	33.1	8.4	7.4	3.2	.4
5th	1.00	9.2	37.5	33.3	8.6	7.5	3.4	.6
9th	1.20	8.8	36.7	33.5	8.7	7.8	3.8	.8
13th	1.35	8.6	36.1	33.5	8.8	8.0	4.0	1.3
24	1.45	8.4	35.8	33.6	8.8	8.1	4.2	1.2
Three-months-old distillate								
1st		10.2	38.4	31.6	8.2	6.8	4.4	.4
2nd		9.2	36.6	31.6	8.8	8.2	5.1	.5
3rd		9.0	36.8	32.3	8.9	8.0	4.5	.5
4th		8.6	36.0	32.9	9.0	8.4	4.6	.4
5th		8.3	35.6	33.2	9.1	8.8	4.6	.5
9th		8.0	35.0	33.6	9.1	9.0	4.8	.5
13th		7.9	34.7	33.8	9.1	9.2	4.8	.5
24		7.7	34.5	33.9	9.1	9.2	5.0	.5
Ether extract		7.0	31.4	35.0	10.2	11.1	3.1	2.1

Table 14.--Monoterpene composition of the first and second 24-hour flow of ponderosa pine resin

Tree number	24-hour period	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown
		Percent						
1-IFG	1st	8.9	34.4	30.9	9.5	10.0	4.0	2.3
	2nd	8.2	34.9	35.8	8.9	8.3	1.9	2.1
2-IFG	1st	5.0	14.6	51.9	11.2	13.1	1.9	2.3
	2nd	5.4	13.8	52.2	10.8	12.9	2.7	2.3
3-IFG	1st	6.7	34.3	38.2	9.5	7.1	2.1	2.1
	2nd	7.2	31.8	37.0	10.5	8.0	3.1	2.5
12-IFG	1st	6.1	31.6	44.3	9.4	7.1	.5	1.0
	2nd	5.8	31.1	40.9	9.3	8.9	1.6	2.4
5-IFG	1st	8.0	41.0	20.4	11.5	16.4	1.5	1.2
	2nd	8.2	42.4	21.1	10.6	15.3	1.6	.8

Table 15.--Analysis of two types of sample preparation of ponderosa pine terpenes at two times after preparation of sample

Tree	Type of preparation	Time of analysis[1/]	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown
			Percent						
1	Distillate	(a)	5.3	14.4	50.5	11.2	12.3	2.1	4.2
		(b)	6.2	15.6	52.7	10.0	11.7	1.0	2.7
2	Distillate	(a)	7.8	35.5	35.2	8.6	9.4	1.6	2.0
		(b)	7.7	36.0	36.0	8.6	9.3	1.4	1.0
3	Distillate	(a)	8.0	41.4	20.7	11.9	15.3	1.9	0.8
		(b)	8.2	42.4	21.1	10.6	15.3	1.6	0.8
4	Distillate	(a)	12.2	52.0	trace	17.2	16.8	1.8	0.0
		(b)	12.3	52.0	trace	16.5	17.1	2.1	0.0
5	Distillate	(a)	5.6	21.4	26.5	24.1	19.1	1.8	1.6
		(b)	5.5	21.9	26.5	23.3	19.2	1.4	1.9
6	Distillate	(a)	3.8	13.8	26.5	25.5	25.5	3.0	1.9
		(b)	4.1	15.0	27.1	24.2	26.5	1.8	1.2
7	Extract	(a)	4.1	17.4	30.2	15.1	31.4	1.7	trace
		(b)	3.4	16.7	30.7	14.8	31.8	1.5	1.1
8	Extract	(a)	6.1	27.6	27.3	12.2	21.2	4.1	1.5
		(b)	5.3	29.6	29.6	12.1	20.4	2.9	trace
9	Extract	(a)	6.2	36.2	44.1	6.4	5.3	1.8	trace
		(b)	6.8	36.8	43.4	5.5	4.8	.9	1.8
10	Extract	(a)	14.1	55.2	trace	28.0	trace	2.8	0.0
		(b)	12.4	59.3	trace	26.0	1.2	1.2	0.0
11	Extract	(a)	8.4	38.1	34.3	12.5	2.9	2.9	0.9
		(b)	7.1	39.2	36.5	11.6	2.0	2.0	1.6
12	Extract	(a)	3.3	5.4	64.2	9.2	12.9	2.5	1.7
		(b)	2.7	3.6	70.5	7.6	11.6	1.8	2.2

[1/] (a) Immediately after preparation of sample.
(b) Four to six months after preparation.

(table 16) show practically no difference around the tree and but slight differences between the 3- and 60-foot height. The slight differences between the two heights could be real, or they could result from variations which do occur in the sample preparation, analysis, and calculations.

Table 16.--Monoterpene composition of a ponderosa pine resin at two trunk heights and four compass points around the tree

Compass position	α-pinene	β-pinene plus myrcene	Δ_3-carene	Limonene plus β-phellandrene
		Percent		
60-ft. height				
N	7.1	45.9	38.4	8.6
E	7.4	45.3	39.9	7.5
S	7.4	46.7	39.9	6.0
W	8.2	45.8	38.4	7.6
$\bar{x}$	7.5	45.9	39.2	7.4
3-ft. height				
N	5.7	49.3	40.2	4.8
E	7.0	49.1	39.6	4.3
S	7.3	47.6	40.1	4.9
W	6.9	47.3	39.7	6.9
$\bar{x}$	6.7	48.3	39.7	5.2

Five trees at Joseph Creek were tapped at 3 and at about 15 feet above ground. The results of the analysis (table 17) show practically no differences between the two positions.

Table 17.--Monoterpene composition of the resin of ponderosa pines at two trunk heights

Tree number[1/]	Height	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown
	Feet				Percent			
3	15	5.7	35.2	52.9	3.1	.4	.9	1.8
	3	5.5	34.7	52.0	3.6	.7	.9	0.0
10	15	6.6	23.3	48.5	8.3	7.5	4.4	1.5
	3	5.0	29.1	48.3	6.8	6.3	1.3	3.1
14	15	1.2	0.0	86.3	11.8	0.0	tr	tr
	3	1.0	0.0	86.7	9.4	1.5	.5	1.0
56	15	7.8	40.8	34.0	12.1	1.9	1.9	1.5
	3	7.6	40.0	37.4	11.3	1.4	2.3	tr
Ch. 1	15	8.1	17.1	46.1	9.7	11.0	4.5	3.5
	3	6.4	17.5	52.1	7.9	10.7	2.5	2.9

[1/] Joseph Creek.

Seven trees at Sly Park were tapped on the north and south side at a 3-foot height. Again the analyses (table 18) show little difference between the two sides of the trees.

Within a Season

In 1961 tree No. 1 at LFG was tapped periodically from June to September, and the monoterpenes were obtained by molecular still distillation at 40° C. for 24 hours. No gas chromatograph was available at the time, so the samples were kept in refrigeration until April of 1962 when they were analyzed on an Aerograph A-90-C with a 6-foot DDP column. Certain separations were not sharp, but it was apparent that there were no radical seasonal changes in the monoterpene composition. The samples were analyzed with a different model chromatograph (the A-90-P) with the 20 percent ODPN column in December 1962. The results (fig. 3) show very little change during the period of sampling.

Table 18.--Monoterpene composition of ponderosa pine resin from the north (N) and south (S) side of the tree

Tree number 1/	Side of tree	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown
		Percent						
1	N	5.5	25.9	38.9	13.0	14.3	1.4	1.0
	S	5.3	26.2	38.3	13.1	14.0	1.6	1.6
2	N	3.1	15.4	38.5	13.6	25.9	2.1	1.4
	S	4.0	17.8	37.4	14.5	22.3	1.2	2.8
3	N	5.8	35.2	25.4	14.4	16.2	1.8	1.2
	S	7.1	33.3	26.0	14.6	15.4	3.7	tr
5	N	6.1	27.6	27.3	12.2	21.2	4.1	1.5
	S	5.3	29.9	29.3	11.7	19.6	3.2	.9
6	N	8.0	25.9	23.5	12.0	27.9	2.8	tr
	S	7.4	29.8	24.7	10.7	26.4	1.0	tr
8	N	6.4	31.3	31.7	12.8	13.6	3.0	1.1
	S	5.0	29.8	33.1	13.6	14.0	3.7	.8
9	N	6.5	35.9	24.9	14.7	15.5	1.1	1.4
	S	7.8	36.9	22.9	14.9	12.0	4.7	.8

1/ Sly Park plot No. 1.

Between Seasons

Eleven trees at IFG were tapped in August 1962 and again in March of 1963 to determine any variation in monoterpene composition between the active and dormant seasons. The results (table 19) show practically no change between the two periods. Changes usually were less than 2 percent except for about a 4 percent shift in β-pinene in tree No. 12. The general trend for a reduction in the percentage of β-phellandrene during the dormant season may be an artifact caused by its low percentage. Further sampling will be needed to more adequately substantiate this conclusion.

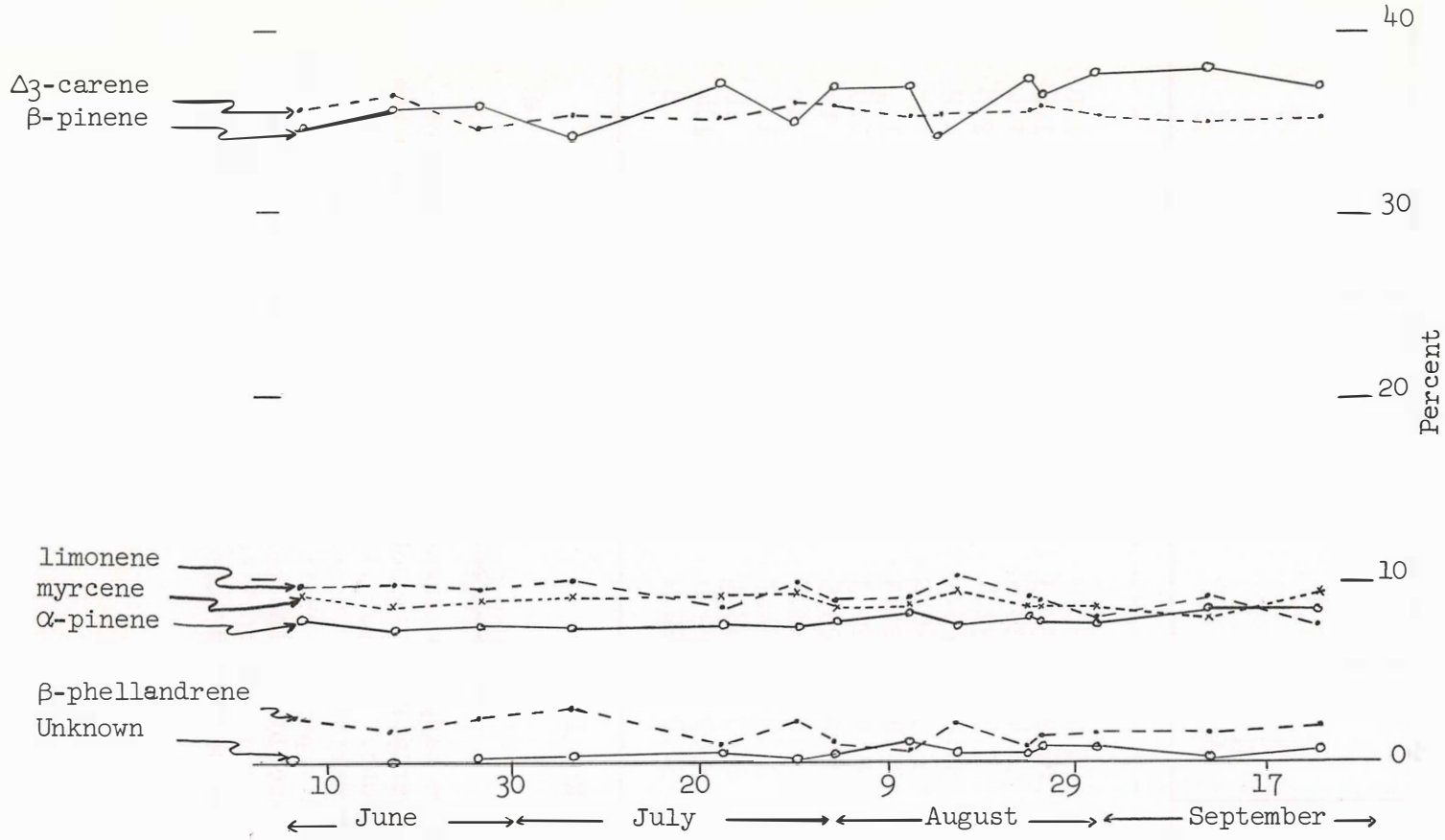

Figure 3.--Seasonal trend in the monoterpene composition of the resin of one ponderosa pine.

Table 19.--Monoterpene composition of ponderosa pine resin obtained during the active and inactive growing season

Tree[1/]	Month	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown
		Percent						
1	August	7.8	35.3	34.7	9.1	9.5	1.7	1.9
	March	6.9	34.5	37.5	8.9	9.6	.9	1.7
2	August	5.8	14.9	50.8	11.0	12.7	2.0	2.8
	March	5.0	14.6	53.6	10.5	12.3	1.0	3.0
3	August	7.9	35.9	37.3	8.6	6.7	1.4	2.2
	March	8.2	35.1	38.2	8.1	7.3	1.3	1.8
4	August	6.6	30.7	35.9	16.0	6.0	2.5	1.6
	March	6.5	33.4	36.6	15.3	5.7	.6	1.9
5	August	7.9	41.0	21.2	11.1	16.2	1.7	.9
	March	7.3	40.9	20.1	11.5	18.1	.9	1.1
6	August	6.4	30.1	43.1	5.9	10.4	1.7	2.4
	March	7.3	32.6	40.0	5.1	12.6	1.3	1.1
7	August	7.9	34.9	38.9	5.2	10.1	1.4	1.6
	March	7.1	32.9	41.2	5.8	10.4	1.0	1.6
8	August	5.7	25.8	44.5	6.8	13.6	1.6	2.0
	March	5.8	27.9	42.6	6.6	14.0	1.3	1.8
9	August	7.7	8.9	65.9	8.5	5.0	.9	3.1
	March	7.1	9.4	62.7	9.7	6.5	1.2	3.4
11	August	7.2	35.3	37.4	9.2	7.5	1.6	1.8
	March	6.7	35.0	36.2	10.9	8.4	1.6	1.2
12	August	7.1	29.4	40.9	9.2	9.8	1.6	2.0
	March	6.3	33.3	38.9	10.3	8.6	1.1	1.5

[1/] Institute of Forest Genetics.

Between Years

One sample of resin of the tree No. 1 at IFG had been obtained in 1960 and kept in a tight container until 1961 when it was processed with the molecular still. Samples were also obtained in 1961, 1962, and in 1963. Therefore, it was possible to compare the monoterpene composition of this one tree for 4 years. The data (table 20) show only slight changes over the 4-year period. There does seem to be a trend for an increase in limonene and myrcene with a corresponding decrease in β-pinene. Further sampling will be required before the trends can be determined as real or apparent.

Table 20.--Monoterpene composition of one ponderosa pine in 4 years

Component	July 1960	July 1961	July 1962	March 1963
	- - - - - - - - Percent - - - - - - - -			
α-pinene	8.4	7.3	7.4	7.0
β-pinene	39.1	35.8	33.8	33.6
Δ_3-carene	36.8	36.7	34.4	37.5
Myrcene	7.1	8.6	9.7	9.4
Limonene	7.3	9.9	10.0	9.9
β-phellandrene	.7	1.7	2.1	1.0
Unknown	.6	t	2.7	1.6

BETWEEN-TREE VARIATION

With little or no variation attributable to procedure, within-tree position, season of sampling, and year of sampling, it was possible to compare ponderosa pines with reasonable assurance that differences between trees were real.

Throughout the course of the season resin from groups of ponderosa pines in eight plots (fig. 4) was obtained, and its monoterpene composition analyzed. Data of this kind could show local types of ponderosa pine stands, and as the results in table 21 indicate, there was considerable variation in plot means of different monoterpenes. However, more extensive sampling will be needed to establish that this is due to real local differences, especially in view of the very great range of variation between trees within a plot.

Plot	Number of trees	Use of trees: Forced attack	Vapor toxicity studies	Within-tree variation	Tree age study	Phenotypic resistance	Type of stand
IFG	11	*	*	*			2nd growth
Sly Park #1	13	*		*			2nd growth
Sly Park #2	6	*					2nd growth[1/]
Riverton	6				*		Overmature
Kyburz	7				*		Overmature
Pyramid	3				*		Overmature
Crystal Bay	12					*	2nd growth
Joseph Creek	7			*		*	2nd growth

1/ Much younger than Sly Park No. 1 and about 50 yards away.

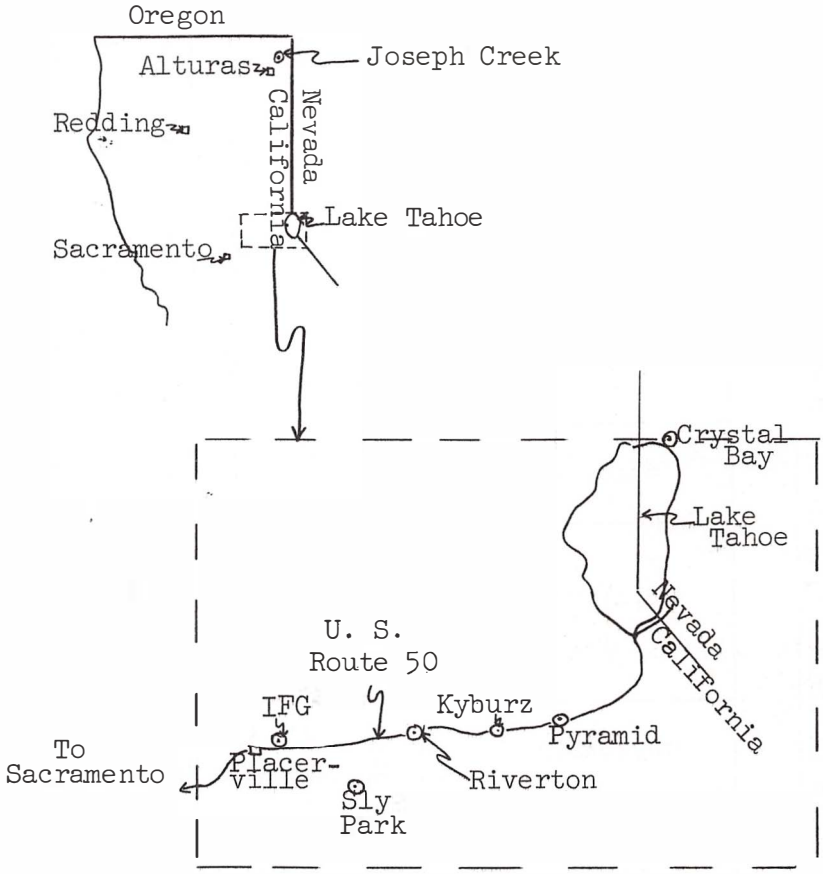

Figure 4.--Location of ponderosa pines used for gas chromatographic analyses of terpenes.

Table 21.--Terpene composition for average ponderosa pine resin in eight plots in the central Sierra Nevada and Warner Mountains

Plot	Trees sampled	Elevation	Stand condition	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown
	Number	Feet	Type of stand	Percent						
IFG	11	2700	2nd growth	7.1	29.4	40.9	9.2	9.8	1.6	2.0
Sly Park No. 1	12	3500	2nd growth	6.0	26.5	31.4	13.7	18.9	2.4	1.1
Sly Park No. 2	6	3500	Pole	7.1	31.5	24.6	12.9	20.5	2.1	1.3
Riverton	6	3000	Mature	7.9	30.4	25.4	17.5	16.0	1.9	.9
Kyburz	7	4000	Mature	6.0	24.9	28.8	17.4	19.8	1.8	1.3
Pyramid	3	5000	Mature	4.4	18.2	46.8	14.1	13.0	1.7	1.8
Crystal Bay	12	6000	2nd growth	5.2	22.3	42.4	13.3	13.4	1.8	1.6
Joseph Creek	7	4000	2nd growth	6.2	25.9	48.6	11.1	4.7	1.8	1.7
$\bar{X}$				6.3	26.4	36.2	13.3	14.5	1.8	1.5

Efforts were made to determine the effect of age of tree on terpene composition. The plots at Riverton, Kyburz, and Pyramid consisted of overmature trees greater than 200 years old; all remaining plots contained young, second-growth trees with ages ranging from 20 to 75 years. No sound conclusion can be drawn from the data. The results from the Sly Park plots are not appreciably different from those for the three old-aged plots, while the IFG plot is quite different, particularly with respect to greater Δ_3-carene and less myrcene and limonene than the three old-aged plots.

From the data in the three overmature plots one might consider a cline with the increase in elevation from Riverton to Pyramid, since β-pinene decreases and Δ_3-carene increases from Riverton to Pyramid through Kyburz (roughly from 3,000 to 5,000 feet elevation). However, the data for IFG, the lowest elevation, are not too different from those for Pyramid, the highest elevation. Again, the need for more extensive sampling is indicated before sound conclusions can be drawn.

There are many ways to present the average values for each of the trees and plots tabulated in table 22. One rather concise way is to show the monoterpene composition of the average ponderosa pine together with the maximum and minimum values found for each of the terpenes (fig. 5). Two sets of maximums and minimums are shown in this figure; one set, the x-line, represents all the data; the other set, the y-line, includes all the data except those for Crystal Bay and Joseph Creek plots. This figure shows quite clearly that any one of the five major components (α-pinene, β-pinene, Δ_3-carene, myrcene, limonene) may comprise less than 5 percent of the total monoterpene. Of considerable interest is the possible presence in a given tree of only a faint trace; i.e., less than 1 percent of β pinene, Δ_3 carene, and limonene. Mirov (1961) considers Δ_3-carene the trademark of ponderosa pine, yet three trees were found which lack it completely or contain only trace amounts. Of considerable entomological interest is the possible presence of as much as 30 percent limonene, the terpene which caused the greatest mortality to both D. brevicomis and D. monticolae in vapor toxicity tests.

The wide range in the monoterpene composition of ponderosa pine is illustrated in figure 6, which depicts the data for selected trees in the 65-tree sample. Tree No. 5-IFG has a large percentage of β-pinene and a moderate amount of Δ_3-carene. Trees No. 9-3 Sly Park, No. 4 Riverton, No. 4 Ch. J. Creek also have large percentage of β-pinene but have only traces of Δ_3-carene. On the other hand, tree No. 14-J.Creek has a large percent of Δ_3-carene, no β-pinene, only a trace of limonene and an unusually large amount of myrcene. Two other trees No. 9-IFG and No. 1c-Crystal Bay have large percentages of Δ_3-carene and greatly reduced amounts of β-pinene. Tree No. 1c-Crystal Bay has no limonene.

Table 22.--Average monoterpene composition for ponderosa pines

Tree number	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown	Tree use
	Percent							
Institute of Forest Genetics								
1	7.8	35.3	34.7	9.1	9.5	1.7	1.9	1/ 2/ 8/
2	5.8	14.9	50.8	11.0	12.7	2.0	2.8	2/ 8/
3	7.1	29.4	40.9	9.2	9.8	1.6	2.0	3/ 8/
4	6.6	30.7	35.9	16.0	6.7	2.5	1.6	8/
5	7.9	41.0	21.2	11.1	16.2	1.7	.9	8/
6	6.4	30.1	43.1	5.9	10.4	1.7	2.4	8/
7	7.9	34.9	38.9	5.2	10.1	1.4	1.6	4/ 8/
8	5.7	25.8	44.5	6.8	13.6	1.6	2.0	4/ 8/
9	7.7	8.9	65.9	8.5	5.0	.9	3.1	4/ 8/
11	7.2	35.3	37.4	9.2	7.5	1.6	1.8	4/ 8/
12	6.5	30.9	40.4	9.9	8.9	1.5	1.9	4/ 8/
$\bar{X}$	7.1	29.4	40.9	9.2	9.8	1.6	2.0	
Sly Park No. 1								
1	5.3	26.3	38.5	12.8	14.0	1.6	1.5	4/ 3/
2	3.8	17.2	37.9	13.6	24.1	1.5	1.9	4/ 3/
3	6.2	34.9	26.5	14.0	15.0	2.6	.8	4/ 3/
4	4.5	17.2	29.6	15.4	30.7	1.8	.8	4/
5	5.7	29.4	28.7	11.9	20.3	3.1	.9	4/ 3/
6	7.1	27.5	24.2	11.5	27.4	2.1	.2	4/ 3/
7	6.9	24.3	38.8	16.4	11.0	1.3	1.3	4/
8	6.2	31.9	31.9	12.9	13.1	3.0	1.0	4/ 3/
9	7.5	37.3	23.4	14.7	13.4	3.0	.7	4/ 3/
10	6.5	23.4	36.7	11.7	17.5	2.2	2.0	4/
25-1	6.8	30.3	22.3	20.2	16.5	2.9	1.0	4/
-2	5.5	16.9	49.2	8.5	15.5	2.3	2.1	4/
-3	6.3	24.9	25.7	12.6	25.3	3.7	1.5	4/
$\bar{x}$	6.0	26.3	31.8	13.6	18.8	2.4	1.2	

Table 22.--Continued

Tree number	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown	Tree use
				Percent				
				Sly Park No. 2				
1	8.4	31.8	24.8	18.2	13.3	2.4	1.1	4/
2	5.0	24.0	38.5	9.0	20.1	1.5	2.0	4/
3	4.8	18.9	32.6	14.7	24.5	2.3	2.2	4/
4	5.8	29.3	28.1	5.9	28.3	1.3	1.3	4/
5	7.2	29.6	23.2	13.8	21.0	3.5	1.7	4/
6	11.5	55.3	.4	15.5	15.5	1.8	--	4/
$\bar{x}$	7.1	31.5	24.6	12.9	20.5	2.1	1.3	
				Riverton				
1	5.7	20.7	38.2	18.4	13.8	1.4	1.8	5/
2	9.3	34.9	22.2	20.4	10.5	2.4	.3	5/
3	8.7	21.4	28.8	14.4	23.2	2.4	1.1	5/
4	11.9	51.2	t	16.6	18.2	2.1	--	5/
5	5.5	25.3	35.5	16.8	13.4	2.0	1.5	5/
6	6.1	28.6	27.7	18.3	16.9	1.4	1.0	5/
$\bar{x}$	7.9	30.4	25.4	17.5	16.0	1.9	.9	
				Kyburz				
1	6.7	29.3	19.6	15.7	25.8	1.8	1.1	5/
2	7.6	36.9	33.3	7.4	12.6	1.5	.7	5/
3	5.6	21.4	27.3	23.3	19.5	1.5	1.4	5/
4	4.0	14.4	27.3	24.9	26.5	1.8	1.1	5/
5	6.3	25.7	21.9	19.1	24.3	1.7	1.0	5/
6	6.2	21.8	38.9	14.8	13.2	2.5	2.6	5/
7	5.4	24.8	33.1	16.5	17.1	1.6	1.5	5/
$\bar{x}$	6.0	24.9	28.8	17.4	19.8	1.8	1.3	

Table 22.--Continued

Tree number	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown	Tree use
	- - - - - - - - - - - - - - - - Percent - - - - - - - - - -							
				Pyramid				
1	4.0	16.5	52.6	10.3	12.3	1.9	2.4	5/
4	5.2	20.5	43.2	14.3	14.2	1.3	1.3	5/
5	4.2	17.6	44.7	17.6	12.4	1.8	1.7	5/
$\bar{x}$	4.4	18.2	46.8	14.1	13.0	1.7	1.8	
				Crystal Bay				
1	4.4	12.8	43.6	12.5	22.6	2.8	1.3	6/ 2/
1a	5.3	25.8	40.8	10.3	13.6	2.1	2.1	7/
1b	4.0	20.8	41.2	10.8	19.9	1.3	2.0	7/
1c	6.2	16.7	59.2	15.5	t	.3	2.1	7/
2	4.6	22.4	39.8	13.5	16.4	1.8	1.5	6/
2a	6.1	28.5	33.1	12.5	16.1	2.4	1.3	7/
2b	3.5	11.8	51.0	16.6	12.8	1.9	2.4	7/
2c	3.4	14.5	45.5	16.7	15.5	1.7	2.7	7/
3	7.4	35.9	43.0	6.0	5.3	1.5	.9	6/
3a	6.9	37.7	26.6	10.1	16.6	1.5	.6	7/
3b	5.0	20.0	32.7	26.4	12.4	2.9	.6	7/
3c	5.4	21.2	51.7	8.6	9.9	1.2	2.0	7/
$\bar{x}$	5.2	22.3	42.4	13.3	13.4	1.8	1.6	

Table 22.--Continued

Tree number	α-pinene	β-pinene	Δ_3-carene	Myrcene	Limonene	β-phellandrene	Unknown	Tree use
	- - - - - - - - - - - - - - - - Percent - - - - - - - - - -							
				Joseph Creek				
3	5.8	33.3	50.6	4.6	1.3	1.8	2.6	6/ 3/
10	5.8	27.1	49.0	7.2	6.5	2.3	2.1	6/ 3/
14	1.5	.1	82.5	11.1	1.5	1.4	1.9	6/ 3/ 2/
56	7.7	40.0	35.8	11.6	1.8	2.0	1.1	6/ 3/
Ch-1	6.4	18.8	52.5	7.8	9.6	2.2	2.7	7/ 3/
Ch-4	13.3	57.5	t	27.5	.4	1.3	--	7/
Ch-5	2.8	4.1	69.4	8.2	12.0	1.8	1.7	7/
$\bar{x}$	6.2	25.9	48.6	11.1	4.7	1.8	1.7	
Grand $\bar{X}$	6.3	26.4	36.2	13.3	14.5	1.8	1.5	

1/ Basic vapor toxicity studies.
2/ Toxicity of vapors of molecular distillate.
3/ Within-tree resin variation.
4/ Forced-attack studies.
5/ Age vs. resin study.
6/ Phenotypic resistance.
7/ Check trees for phenotypic resistance.
8/ Seasonal and yearly resin variation.

The remaining trees have moderate amounts of all the four major components, though the percentages do range quite a bit, particularly myrcene. Of passing interest is the similarity between the average ponderosa pine and No. 1-IFG, which was the source of resin for the great bulk of vapor toxicity studies.

There appears to be an inverse relationship between the amounts of β-pinene and Δ_3-carene as shown in figure 7. This relationship is sharpest at the extremes.

CONCLUSIONS

Results with gas chromatography were highly productive and measurably increased our understanding of ponderosa pine resin. The results also helped to explain certain points which were left unresolved in earlier studies. Further studies should be equally as productive.

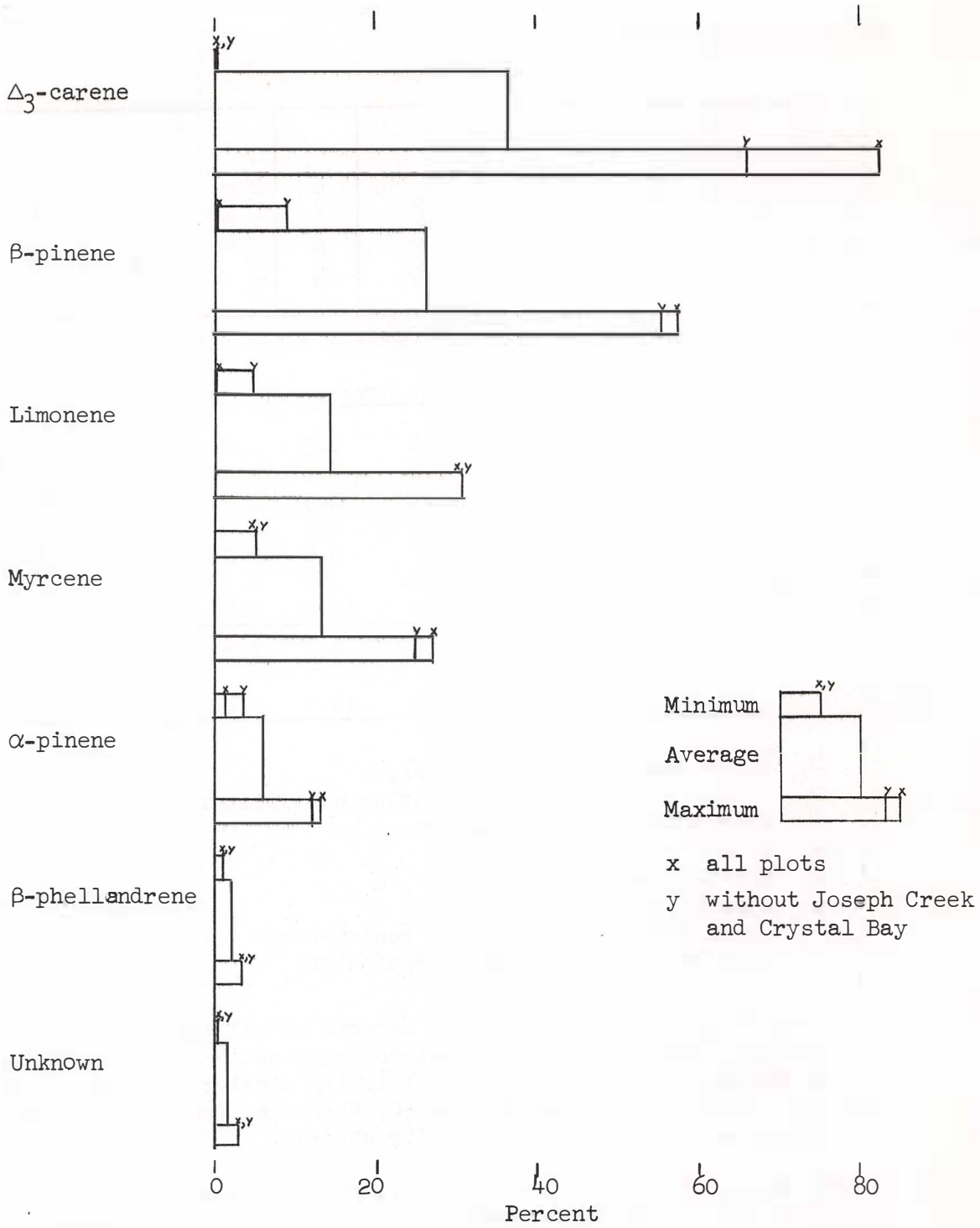

Figure 5.--Variation of individual terpenes in the total terpene of the resin of 64 ponderosa pines.

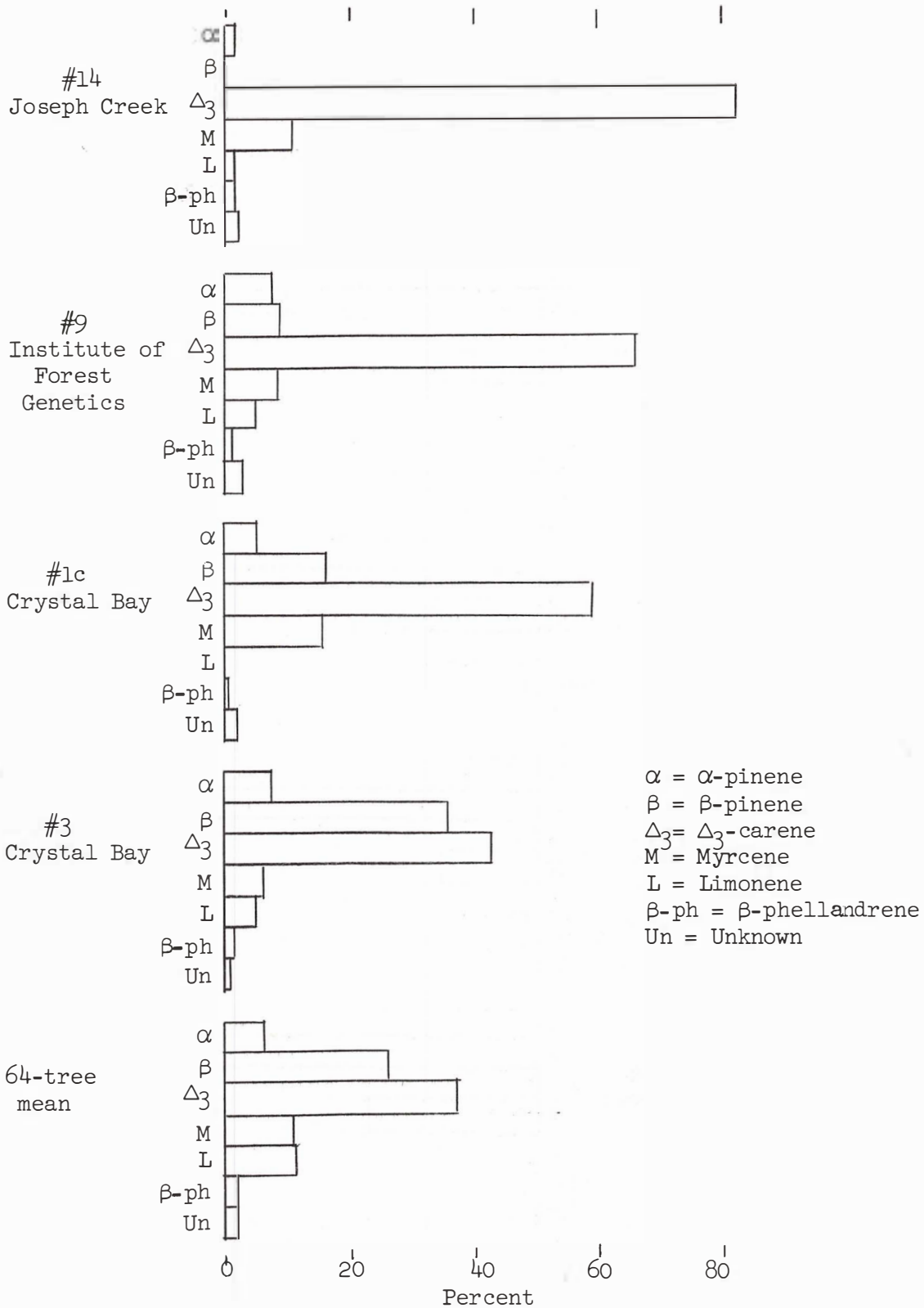

Figure 6.--Terpene composition of selected ponderosa pines.

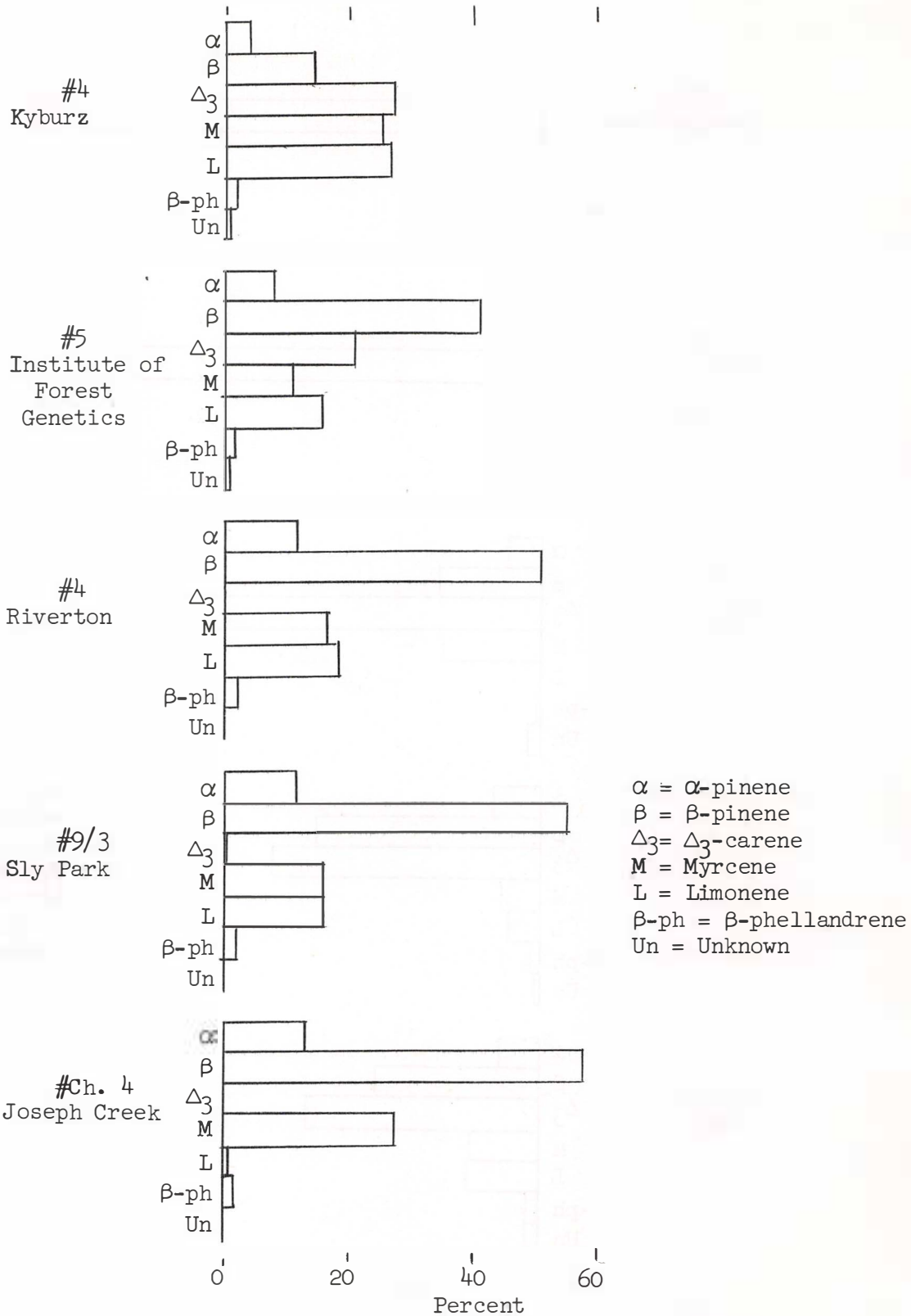

Figure 6 (Con.).--Terpene composition of selected ponderosa pines.

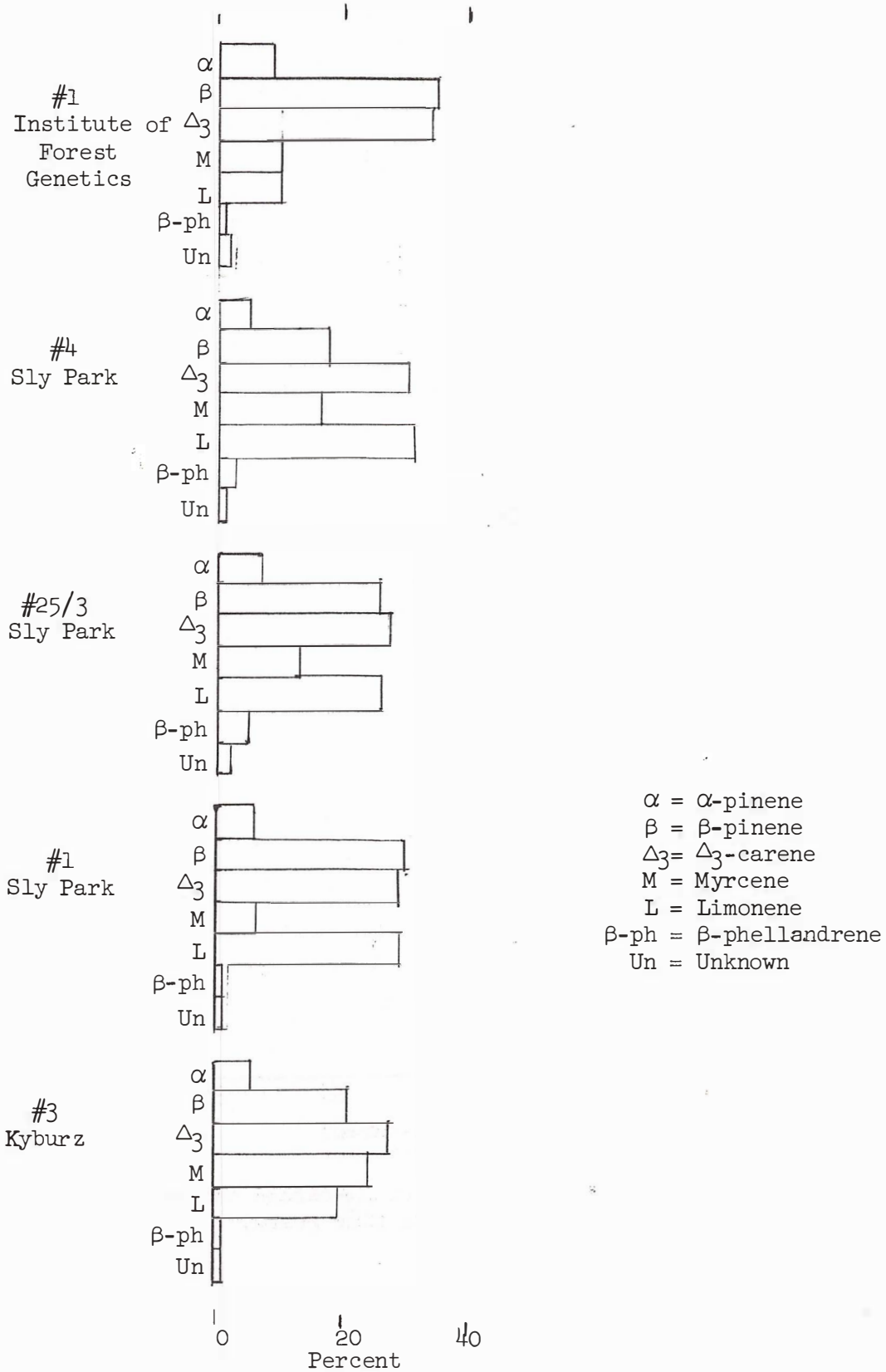

Figure 6 (Con.)--Terpene composition of selected ponderosa pines.

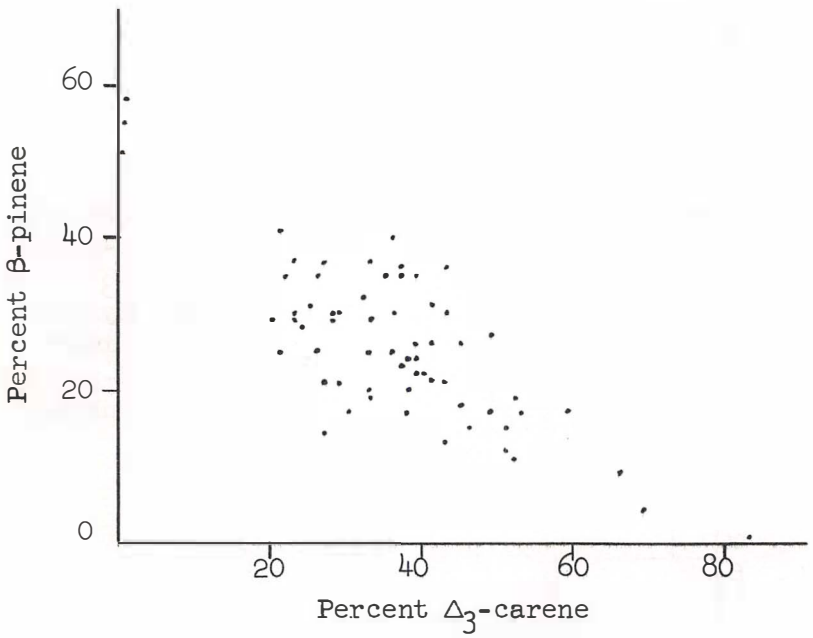

Figure 7.--Relationship of Δ_3-carene and β-pinene in ponderosa pine resin.

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